Vitamin D Prevents Endothelial Damage Induced by Increased Neutrophil Extracellular Traps Formation in Patients with Systemic Lupus Erythematosus

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ABSTRAK

Tujuan: *mengetahui efek pemberian Vitamin D calcitriol/1,25(OH)$_2$D3 pada proses NETosis pasien Systemic Lupus Erythematosus (SLE) dengan kondisi hipovitamin D.* ***Metode:*** *neutrofil dari lima pasien SLE dengan hipovitamin D dikultur dan dibagi menjadi 4 kelompok, P0 (0 nM/kontrol), P1 (1 nM), P2 (10 nM) dan P3 (100 nM). Pembentukan NETs diinduksi dengan pemberian Phorbol Myristate Acetate (PMA). Supernatan dipisahkan dan dikokultur dengan HUVECs. Eksternalisasi Neutrofil Elastase (NE) diukur menggunakan metode imunofloresens dan Myeloperoxidase (MPO) menggunakan metode ELISA selama NETosis. Early apoptosis dan late apoptosis sel endotel diukur dengan metode sitometri alir (flowcytometry) menggunakan cell death kit (antibodi Annexin V dan PI).* ***Hasil:*** *studi menunjukkan adanya penurunan early apoptosis yang signifikan pada kosentrasi 10 nM 1,25(OH)$_2$D3 dibandingkan kelompok kontrol. Eksternalisasi NE signifikan pada semua kelompok perlakuan ($p<0.05$), sementara absorbansi MPO memiliki tendensi yang sama namun tidak signifikan secara statistik. Analisis menunjukkan adanya korelasi positif yang cukup kuat antara eksternalisasi NE dengan early apoptosis.* ***Kesimpulan:*** *vitamin D dapat menurunkan kerusakan endotel melalui penurunan aktivitas NETosis. Hasil dari studi ini dapat membuka peluang penggunaan Vitamin D sebagai terapi suplementer bagi pasien SLE dengan hipovitamin D untuk mencegah kerusakan endotel.*

Kata kunci: *NETs, NE, MPO, 1,25(OH)$_2$D3, HUVECs*

ABSTRACT

Aim: *to investigate the effects of Vitamin D calcitriol/1,25(OH)$_2$D3 on NETosis in systemic lupus erythematosus (SLE) patients with hypovitamin D.* ***Methods:*** *neutrophlis of five SLE patients with hypovitamin D were divided into 4 groups, P0 (0 nM/control), P1 (1 nM), P2 (10 nM), and P3 (100 nM) as cultured samples. Phorbol Myristate Acetate (PMA) was used to stimulate NETs formation. The supernatant was separated and cocultured with HUVECs. Externalization of Neutrophil Elastase (NE) and Myeloperoxidase (MPO) during NETosis was measured by immunofluorescence and ELISA respectively. Early and late apoptosis of endothelial*

cell was measured by flowcytometry using cell death kit (Annexin V and PI antibody). ***Results:*** *this study showed significant decrease in early apoptosis with 10 nM of 1,25(OH)$_2$D3 compared to control group. Significance of NE externalization found in all treatment groups ($p<0.05$), while MPO absorbance in the same tendency but not statistically significant. Further analysis also found a moderate positive correlation between NE externalizations with early apoptosis.* ***Conclusion:*** *vitamin D 1,25(OH)$_2$D3 could reduce endothelial damage by decreasing NETosis activity. This result may reveal the possibility of Vitamin D as supplementary therapy for SLE patients with hypovitamin D to prevent endothelial damage.*

Key words: *NETs, NE, MPO, 1,25(OH)2D3, HUVECs.*

INTRODUCTION

Systemic Lupus Erythematosus (SLE) is a chronic systemic inflammatory autoimmune disease with high morbidity and mortality involving multi organ damage. In Indonesia, life expectancies of SLE patients are still low, 70% for 5 years survival rate and 50% for 10 years survival rate. In recent years, it has been reported that abnormality of neutrophil subsets were identified in peripheral blood mononuclear cell (PBMC) of adult SLE patients. It is shown that low density granulocyte (LDG) has important role in pathogenesis of SLE. It tends to produce neutrophil extracellular traps (NETs) during a distinct form of cell death called NETosis. The process acts as first line of innate immunity as it releases numbers of antibacterial products and chromatins to trap pathogens. NETs is composed of DNA, histone, neutrophil elastase (NE), myeloperoxidase (MPO), human neutrophil protein (HNP) and others granular protein which will be externalized during NETosis.[1] Besides LDGs, SLE patient neutrophils also have ability of producing more NETs. This LDGs and SLE neutrophils capabilities in synthesizing NETs have been shown to give rise at clinical manifestations such as vasculitis and greater production of autoantibodies.[2] Vasculitis itself, is one of rare SLE manifestation, but its presence can be lowering the quality of life of patients even can be life threatening to SLE patients.[3]

Several recent studies found that some components of the NETs were able to directly injure to the endothelium, which in turn cause vasculitis. Neutrophil elastase is neutrophil granular protein acts as antibacterial. It consists of serine proteases which could be activated by inductor, LPS or PMA, to degrade chromatin in forming NETs. Excessive release of NE could also leads to activation of macrophage and chemotactic activity of C5a resulting inflammation.[4] Some researchers stated NE could induce lungs and epithelial damage.[5,6]

On the other hand, there has been widely reported an association between the onset of autoimmune disease with vitamin D deficiency.[7] Various results of the other study states the presence of a high percentage of SLE patients in 4 seasons states with vitamin D deficiency.[8] Our previous study also showed that vitamin D level in SLE patients was significantly low compared to healthy controls. Low levels of vitamin D in the SLE patients was associated with a decreasing function of regulatory T cells, improving function and activities of dendritic cells and B cells which indicated by increasing autoantibodies secretion.[9] Our study reveals the effects of 1,25(OH)$_2$D3 in decreasing endothelial damage during NETosis. It is important because even though Indonesia is located on the equator with sun exposure throughout the year, many patients of SLE in Indonesia suffered from hypovitamin D. SLE patients in Indonesia have more severe clinical manifestation. The results of this study will encourage the importance of vitamin D for SLE patients with hypovitamin D.

METHODS

Sample Preparation

Peripheral venous blood collected from five newly diagnosed female SLE patients (American College of Rheumatology Criteria, 1997), age 17-36 years (mean=26.40±6.77), in active disease state with Mex-SLEDAI score >5, and hypovitamin D (25(OH)D serum level <30 ng/ml), range of 25(OH)D serum level was 16.8

-29.5 ng/ml (mean=21.6±4.74 ng /ml). Subjects divided into 4 groups of 1,25(OH)$_2$D3 doses, P0 (control/0 nM), P1 (1 nM), P2 (10 nM), P3 (100 nM). Thirty mililiters of peripheral venous blood was collected and put in vacutainer with EDTA. Two mililiters of serum from Vacutainer was isolated and used to measure the concentration of 25(OH)D serum by ELISA in accordance with the manufacturer's instructions (NovaTein Bio, USA). Umbilical cord blood was obtained from the placenta of preterm infants (34-36 week gestation) delivered by cesarean section without labor at Saiful Anwar hospital Malang.

Isolation of Neutrophils

Neutrophils from subjects were separated from whole blood using gradient centrifugation with Polymorphprep (Axis-shield PoC AS). Briefly, Collected human venous blood with EDTA carefully placed over Polymorphprep with equal volume, then centrifuge 1400 rpm for 33 minutes at room temperature. Layer of Neutrophils then harvested from lower band and purified from erythrocytes with erythrocyte lysis buffer. Purity was >95%, confirmed neutrophil cells were labeled with CD10 and CD14 positive.

Neutrophil Treatment

Isolated neutrophils was counted with haemocytometer and 1 to 2 x 105 cells/ml was seeded in poly-L-lysine coated coverslip with RPMI 1640 media (Sigma-Aldrich, USA) containing 2 mM glutamin (Gybco, USA), 10 mM HEPES (Gybco, USA), Penicilin-streptomicin 10.000 U/ml (Gybco, USA), and 10% Fetal Bovine Serum (Gybco, USA) at 24 wells. Isolated neutrophils were incubated for 15 minutes at 37^{o}C, 5% CO_2 and treated with different concentration of Vitamin D [1,25(OH)$_2$D3] (Sigma) several doses 1 nM, 10nM, and 100nM for 24 hours.

NETs Production and Isolation

Isolated neutrophils also seeded in poly-L-lysine free 24 wells for NETs production and endothelial cell death assay. Neutrophils were stimulated with 50 nM PMA for 4 hours. Medium was removed and wells were washed with RPMI. PMA, at this concentration, does not promote apoptosis. To collect NET, 2 ml RPMI per well was added and NET (the smear on the wells) was collected by vigorous agitation. After centrifugation at 650 rpm for 5 minutes, NET was collected in the supernatant and stored at –20^{0}C.

HUVECs Culture

HUVECs isolated from human umbilical cord that we collected from cesarean section. Briefly, umbilical cord washed from blood and then collagenase solution inserted to umbilical vein for about 8 minutes. Solution was centrifuged at 1600 rpm for 8 minutes to obtain cell pellet. Isolated HUVECs cultured with serum free which contained Medium 199, gentamicin, phenol red, glutamine, and newborn calf serum (NCS) at 24 gelatin was coated well until confluence. Culture incubated at 37°C and 5% CO_2. Purity of isolated cells (>99%) was assessed by FACS following labelling the cells with endothel-specific marker CD146 (Biolegend).

NETs Immunofluorescence Assay

Treated neutrophil culture was stimulated using 20 nM PMA (Sigma) to generate NETs then incubated for 2 hours. Then culture was washed with ice-cold PBS and fixed with paraformaldehyde for 15 minutes. Fixed cells were treated with triton 0,5% for 1 minute followed by blocking buffer FBS 5% for 30 minutes. Then stained with rabbit anti-human elastase (1:350) for 60 minutes at 37^{o}C followed by incubation with secondary fluorochrome-conjugated antibody FITC donkey anti rabbit (1:500), for 60 minutes. Nuclear DNA was detected using Hoechst 33342 (1:100) incubated for 10 minutes at room temperature. Coverslips were mounted in Prolong Antifade Reagent (Invitrogen) then analyzed using an Olympus microscope. The percentage of NETs was calculated as the average of three to four fields (x40) and quantified as the color intensity using image RGB analysis. Externalization of NE at NETs (colocalizing of NE and DNA) was counted from green intensity per total intensity of the image.

MPO-DNA ELISA Assay

To quantify NETs in cell culture supernatant and in mouse and human plasma, we developed a capture ELISA based on MPO associated with DNA. For the capture antibody, 5 µg/ml anti-MPO mAb (Upstate, catalog no. 07-496) was coated onto 96-well plates (dilution 1:500 in

50 µl) overnight at 4°C. After washing 3 times (300 µl each), 20 µl of samples was added to the wells with 80 µl incubation buffer containing a peroxidase-labeled anti-DNA mAb (Cell Death ELISAPLUS, Roche; dilution 1:25). The plate was incubated for 2 hours, spinning at 300 rpm at room temperature. After 3 washes (300 µl each), 100 µl peroxidase substrate (ABTS) was added. Absorbance at 405-nm wavelength was measured after 20 minutes incubation at room temperature in the dark. Values for soluble NET formation are expressed as percentage increase in absorbance above control.

Cell Death Assay

Supernatant from Netting product of neutrophil (200ul) was cocultured with HUVECs in 24-well plate and incubated for 16 hours. Apoptosis assay was done using flowcytometry (FACScalibur) staining with apoptosis kit (Annexin V–PI). Early and late apoptosis HUVECs was measured respectively in cell with AnnexinV+ PI-, and AnnexinV+ PI+.

Ethical Clearance

This study was approved by the Ethics Committee of Faculty Medicine Brawijaya University Malang and informed consent had been obtained.

Statistical Analysis

Data of NE externalization from immunofluorescence intensity were collected by two blinded observers from Olympus FSX-BSW software. Data of MPO absorbance recorded from ELISA reader. Apoptosis quantification obtained from flowcytometry. All data were analyzed using SPSS 16.0 for Windows. To asses significance among each treatment groups using One way ANOVA, $p<0.05$. Further, correlation between NE and MPO externalization and endothelial apoptosis analyzed with Pearson.

RESULTS

Our sample SLE patients showed no significant difference in age, duration of illness, and level of anti ds-DNA and vitamin D ($p>0.05$). It indicates similarity among the characters of our subjects. Vitamin D levels were 21.60+4.74 ng/mL (**Table 1**), which is below normal level (>30 ng/mL).

Table 1. Characteristics of subjects

Characteristics	SLE Patients (n=5)	p value
Age (mean+SD, years)	26.40+6.77	0.65
Duration of Illness (mean+SD, years)	4.4+2.4	0.78
Anti ds-DNA	167.42+7.86	0.06
Vitamin D level (mean+SD, ng/ml)	21.60+4.74	0.08
Number of LDG (mean+SD, %)	43.39+2.54	0.30
Absorbance of NETs (mean+SD)	0.85+0.15	0.59
Medication History	Methylprednisolone, Cal C, Omeprazole, Folic Acid, Natrium Diclofenac	

Effect of 1,25(OH)$_2$D3 Treatment on the Externalization of NE During NETosis

The 1,25(OH)2D3 treatment in neutrophil culture of SLE patients reduced the externalization of NE during NETosis (**Figure 1**). There was a significant difference in NE externalization (**Figure 2A**) between P1, P2 and P3 compared with P0 (40.83±2.05%; vs. 22.31±1.51%; p=0.004; 40.83±2.05%;vs. 20.48+5.73%; p=0.002 and 40.83±2.05%; vs. 24.61±6.64%; p=0.009). NE externalization was found to be fluctuative as the increasing concentration of 1,25(OH)$_2$D3. This indicated that 1 nM of 1,25(OH)$_2$D3 were able to decrease the externalization of NE during NETosis in neutrophils of SLE patients. However, Vitamin D 100 nM was slightly increase NE externalization. It is shown it trend graph of NE externalization. Statistical analysis revealed there was no significant difference in NE externalization between treatment groups of Vitamin D administration ($p>0.05$).

Effect of 1,25(OH)$_2$D3 Treatment on the Externalization of MPO During NETosis

MPO externalization assay was carried out using ELISA. The result is shown on **Figure 2B**, where the treatment groups slightly lower than the positive control group, despite our data exhibit there is no statistical significance among the treatment groups. However, P2 showed the lowest MPO absorbance, which means less MPO is externalized. 1,25(OH)$_2$D3 treatment decrease the number of MPO externalization.

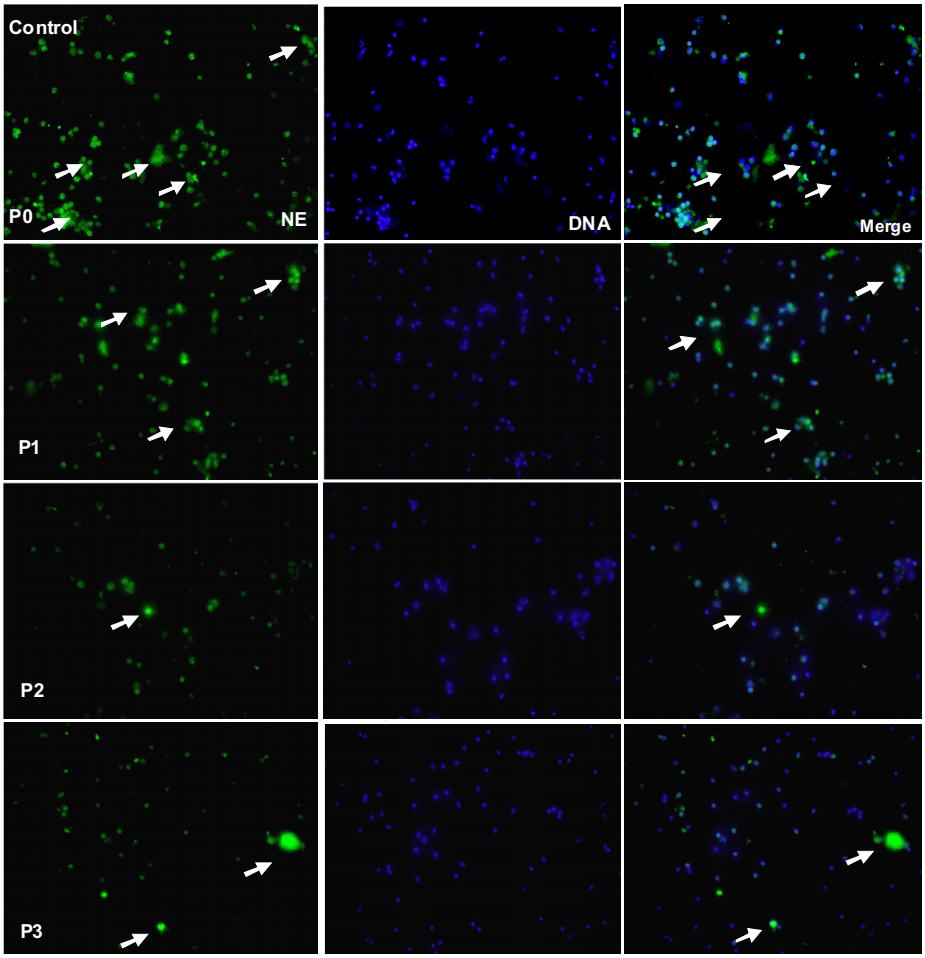

Figure 1. Lupus Neutrophils externalize NE through NETosis. Representative immunofluorescence images of P0, P1,P2, and P3. Cells were stained for detection of neutrophil elastase (green) and DNA (Hoechst 33342, blue). Original magnification 200x.

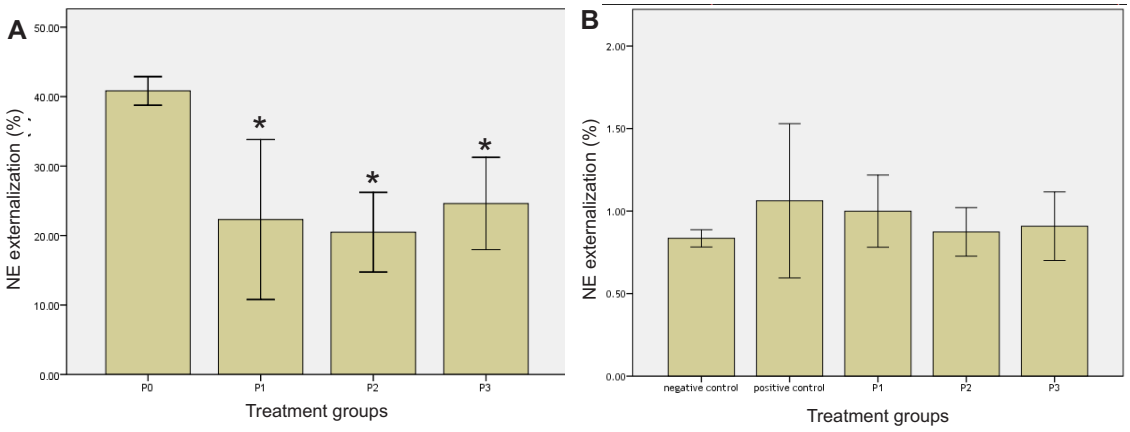

Figure 2. NE externalization during NETosis (A). There were reduced mean of NE externalization among P1 (1nM), P2 (10nM) and P3 (100nM) compared with P0. P2 was the lowest trend compared to control. MPO-DNA externalization during NETosis (B). There were no significantly reduced mean of MPO externalization among any group *Significant ($p<0.05$) compared to control

Effect of 1,25(OH)$_2$D3 Treatment on the Endothelial Early Apoptosis

The 1,25(OH)$_2$D3 treatment in neutrophil culture of SLE patients reduced early apoptosis of endothelial cells due to NETosis as seen in **Figure 3**. There was a significant difference in early apoptosis (**Figure 4A**) between P2 and P0 (2.62±0.53%; vs. 6.17±1.90%; p=0.001). However there was no significant difference in early apoptosis between P1 and P3 compared to P0 (4.61±0.28% vs. 6.17±1.90%; p=0.266 and 3.97±0.59% vs. 6.17±1.90%; p=0.069). This indicated that only 10 nM of 1,25(OH)$_2$D3 were able to decrease the early apoptosis due to NETosis in neutrophils of SLE patients.

However, 1,25(OH)$_2$D3 treatment in neutrophil culture of SLE patients did not reduced late apoptosis. There was no significant difference between P0 and treatment groups P1, P2, P3 (1.00±0.65% vs. 1.57±0.15%, 0.83±0.29%, and 1.57±0.45%, p=0.144) (**Figure 3B**). This results indicated that 1,25(OH)$_2$D3 was not able to decrease the late apoptosis due to NETosis in neutrophils of SLE patients.

In this study, there was significant moderate positive correlation between early apoptosis and NE externalization (r=0.617, p=0.033). This result indicated that treatment of 1,25(OH)$_2$D3 can reduce early apoptosis during NETosis in neutrophils of SLE patients, through inhibition of NE externalization.

DISCUSSION

Recent evidence from various studies indicates that NET formation may be an important phenomenon in autoantigen modification and exposure to the immune system, as well as in the induction of tissue damage.[10,11] As such, extensive NET formation may play an important role in the development and progression of autoimmune

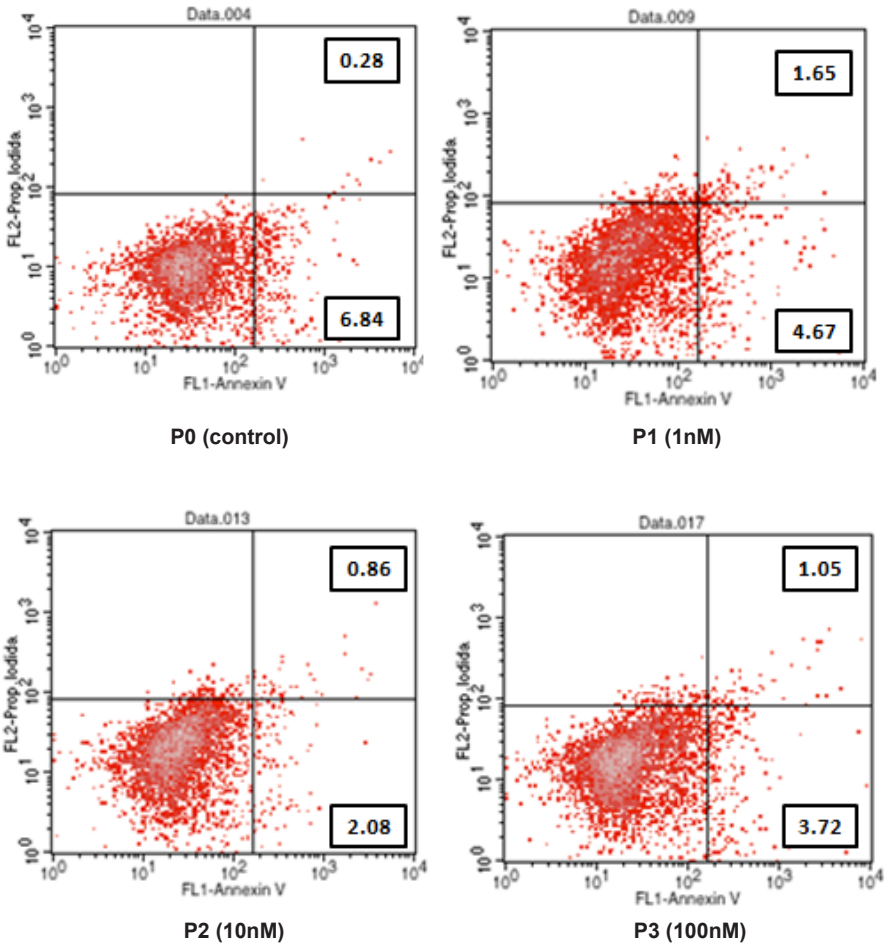

Figure 3. The 1,25(OH)2D3 decreased in early apoptosis. Percentage of early apoptosis showed at lower right quadrant. Percentage of Late apoptosis showed at upper right quadrant

diseases and organ damage observed in chronic inflammatory disorder.[1] Neutrophils found in SLE patients (LDGs) have enhanced capacity to form NETs and upregulate expression of various neutrophil proteins and enzymes implicated in NET formation and autoimmunity induction. These NETs also expose dsDNA, an autoantigen considered key in lupus pathogenesis. NETs itself is composed of DNA, histone, neutrophil elastase (NE), myeloperoxidase (MPO), human neutrophil protein (HNP) and others granular protein which will be externalized during NETosis.[1]

NETosis is demonstrated by challenging neutrophil with PMA, following mechanism of chromatin decondensation and releasing granule contents of neutrophil. This resembles a reaction when pathogen is found adjacent to neutrophil. The process started when NE and MPO are released from azurophil granule and migrate to nucleus causing decondensation of chromatins. The next process is neutrophil burst, externalizing neutrophil granular proteins to form a trap toward pathogens. One most abundant externalized product of NETs is neutrophil elastase. Excessive externalization of NE may cause damage to surrounding cell, including endothelial and epithelial cells. According to a study by Villanueva, NETosis could induce damage to endothelial cell.

It is known that Vitamin D is able to act as immunomodulator. Receptor for vitamin D (VDR) and Vitamin D Binding Protein (DBP) molecule have been discovered in neutrophil which are thought to be responsible in neutrophil response toward circulating Vitamin D molecules.[4] $1,25(OH)_2D3$ was proven to reduce the production of inflammatory mediators and reactive oxygen intermediates in neutrophils through induction of 5-LOX (5-Lipoxygenase) gene and suppressed COX-2 (Cyclooxygenase-2) gene.[15] In another study, $1,25(OH)_2D3$ treatment on macrophages was able to decrease its activity due to inhibition of transcription listericidal protein coding genes, gp91 Phox (Cybb) which is a component of the phagocytic oxidase (Phox) or also called NADPH oxidase.[12] PHOX itself is a key enzyme in the formation of the NETs, PHOX change oxigen into reactive oxigen species wich is a precursor for MPO synthesis.[13] In addition $1,25(OH)_2D3$ also capable to inhibit PI3K/AKT/mTOR pathway through its interaction with VDR and its capability to increase transcription rate of PTEN (Phosphatase and Tensin Homolog) and DDIT4 (DNA - damage - inducible transcript 4).[14] On the other hand, inhibition of the mTOR pathway can reduce the ability to kill bacteria through inhibition of NETs formation.[16] Some of the above mechanisms might explain how $1,25(OH)_2D3$ has the potential to reduce the occurrence of NETosis process. Our data indicated that administration of Vitamin D in different concentration may decrease the intensity of NE externalization, proven statistically significant

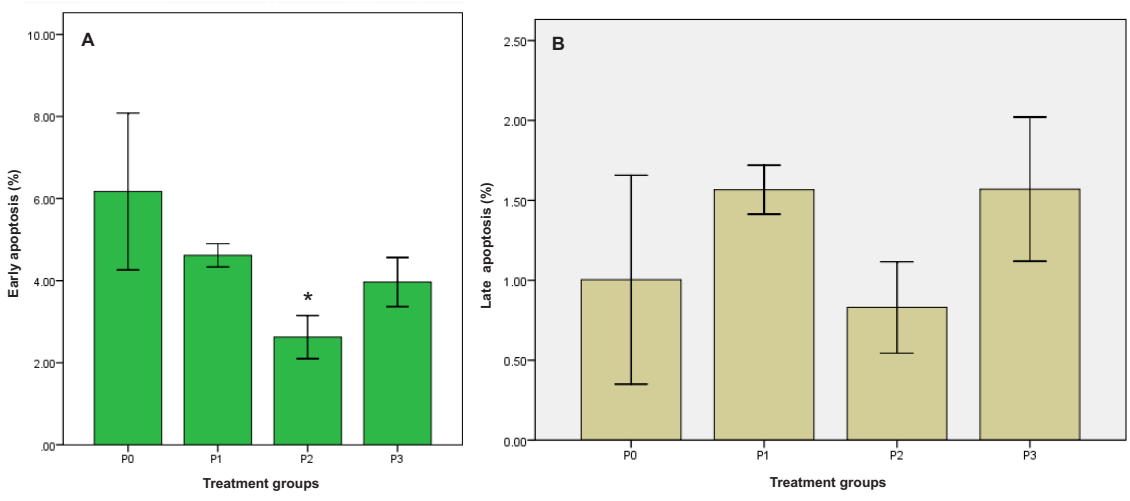

Figure 4. Early apoptosis of endothelial cells during NETosis (A). There were reduced mean of early apoptosis among P2 compared with P0. *Significant ($p<0.05$). Late apoptosis percentage (B). No significant result among each treatment groups.

moderate correlation between concentration of Vitamin D and NE externalization (r=-0.550, p=0.027).

Recent studies show that NE and MPO are granular protein externalized in the same time during NETS.[1,13] But little do we know about the amount of the NE and MPO that externalized. Our study showed that MPO and NE did not externalized at the same amounts. There was a significant changes in NE externalization with administration of $1,25(OH)_2D3$ but not MPO. $1,25(OH)_2D3$ did not decrease the externalization of MPO as significantly as with NE. However MPO has similar tendency to decrease as NE with administration of $1,25(OH)_2D3$ 10 nM. This finding need a further study.

Excessive NE externalization will cause more damage to endothelial cell. This is possibly due to serine protease property which degrades ECM causing soft tissue destruction as in severe inflammatory disease.[5] Some mechanisms, correlated to the high cationic properties of NE will alter endothelial function.[17] Furthermore, Human Neutrophil Elastase (NE) has capability to activate proapoptotic signaling pathway as it enters endothelial cell. The mechanism of activation, together with proteinase 3, is through in vivo cleavage of NF-kB and proapoptotic changes in ERK, JNK and p38 MAPK.[18]

MPO itself may cause toxic effect. In NETosis event, MPO requires presence of hydrogen peroxide as a reagent. The reaction produces hypohalous acids, such as hypochlorous acid (HOCl).[13] HOCl is known to be toxic to endothelial cell. This product may induce the apoptosis of endothelial cell.[19] In this study also found a positive moderate correlation between early apoptosis with NE externalization. This indicated that an increase in externalizing of NE can increase the likelihood of early apoptosis, and vice versa. This result has similarity with the research conducted by Villanueva using SLE patients LDGs.

Administration of Vitamin D is shown to decrease the number of early apoptosis in HUVECs with concentration of 10 nM. The mechanism is possibly through inhibition of NE externalization. Based on our findings the lower the NE is externalized, the less early apoptosis of HUVECs occurred. Vitamin D could inhibit Fas-ligand induced apoptosis in osteoblast through regulation of mitochondrion and Fas-related pathway.[20] We expected the similar mechanism in HUVECs as endothelial cells also expressed Fas Ligand.

Our results showed no significant difference of late apoptosis in three groups of Vitamin D dose. This can be caused by several mechanisms. PI dye that can recognize the cell DNA as a sign of late apoptosis in this study may not be able to pass through the cell membrane that is still quite intact.[21] Apoptotic turnover rate itself depends on the stimulus used to induce apoptosis, while the type of the cell determines the way of the transition within the apoptotic cascade.[22] Histones, NE and MPO act as agents which are suspected to induce cells death in this study may not be able to damage the integrity of the cell at 16 hours of exposure as in previous studies.[1]

The optimal vitamin D levels in serum was >75 nM (>30 ng/ml).[23] Once the nutrient response threshold has been reached, further intake will be no longer benefit. In this study, $1,25(OH)_2D3$ treatment with 100 nM had less significant result than 10nM in decreasing NE externalization and did not show significant decrease in early apoptosis. This condition indicated that after $1,25(OH)_2D3$ reached the optimal or threshold dose, even if $1,25(OH)_2D3$ was given in higher dose, it wouldn't give an optimal result; $1,25(OH)_2D3$ treatment skewed back in result as seen in control. Dose (1 nM) equals with 0 .4 ng/ml[23], so 10-7 M (100 nM) equals with 40 ng/ml. If this dose (100 nM) is given to treat culture, it probably would give a toxic/opposite effect. Sudden 'stressor' in cell culture will make cell give another respond to maintain its homeostasis. Metabolite of 25(OH)D in human should change to $1,25(OH)_2D3$ to become active. The active metabolite, $1,25(OH)_2D3$ will bound to VDR, nuclear receptor that regulate transcription of vitamin D targets gene.[24,25]

SLE patients have greater tendency to suffer from vitamin D deficiency due to lack of sun exposure. The result of this research should expand vitamin D as supplementary therapy especially to SLE patients where sun exposure is less abundant. By administering vitamin D to

hypovitamin D patients might suppress NETosis and prevent vasculitis initiated by endothelial damage thus will improve the quality of life of SLE patients.

CONCLUSION

Administration of vitamin D $1,25(OH)_2D3$, especially dose of 10 nM, may decrease number of endothelial cell early apoptosis through inhibition of externalized NE. Increasing of the dose may give inversely result due to toxic/ opposite effect.

ACKNOWLEDGMENTS

We thank Dr. dr. Loeki Enggar Fitri, M.Kes, Sp.Park for guiding us during ELISA assessment. Also we thank Prof. Dra. Fatchiyah, M.Kes, PhD for helping with immunofluorescence analysis.

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